Identification and characterization of a cluster of genes involved in biosynthesis and transport of acinetoferrin, a siderophore produced by Acinetobacter haemolyticus ATCC 17906^T

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Acinetobacter haemolyticus ATCC 17906^T is known to produce the siderophore acinetoferrin under iron-limiting conditions. Here, we show that an operon consisting of eight consecutive genes, named acbABCD and actBCAD, participates in the biosynthesis and transport of acinetoferrin, respectively. Transcription of the operon was found to be iron-regulated by a putative Fur box located in the promoter region of the first gene, acbA. Homology searches suggest that acbABCD and actA encode enzyme proteins involved in acinetoferrin biosynthesis and an outer-membrane receptor for ferric acinetoferrin, respectively. Mutants defective in acbA and actA were unable to produce acinetoferrin or to express the ferric acinetoferrin receptor under iron-limiting conditions. These abilities were rescued by complementation of the mutants with native acbA and actA genes. Secondary structure analysis predicted that the products of actC and actD may be inner-membrane proteins with 12 membrane-spanning helices that belong to the major facilitator superfamily proteins. ActC showed homology to Sinorhizobium meliloti RhtX, which has been characterized as an inner-membrane importer for ferric rhizobactin 1021 structurally similar to acinetoferrin. Compared to the parental ATCC 17906^T strain, the actD mutant displayed about a 35% reduction in secretion of acinetoferrin, which was restored by complementation with actD, suggesting that ActD acts as an exporter of the siderophore. Finally, the actB product was significantly similar to hypothetical proteins in certain bacteria, in which genes encoding ActBCA homologues are arranged in the same order as in A. haemolyticus ATCC 17906^T. However, the function of ActB remains to be clarified.

INTRODUCTION

Iron is a requisite nutrient for the growth and proliferation of the vast majority of microbes. However, iron bioavailability is often limited due to the insolubility of iron in aerobic environments at neutral-to-alkaline pH or to sequesteration of iron by high-affinity iron-binding proteins such as lactoferrin and transferrin within the host to avoid microbial infections through non-specific mechanisms (Ratledge & Dover, 2000; Bullen et al., 2005; Miethke & Marahiel, 2007). Thus, to overcome this iron restriction in the competitive local environment, many microbes have evolved diverse strategies, one of the most prominent being the biosynthesis of low-molecular-mass chelators, called siderophores (Neilands, 1981; Hider & Kong, 2010). They are secreted into the local extracellular milieu, where they bind ferric iron with high specificity. In Gram-negative bacteria, iron-loaded siderophores (ferric siderophores) are transported back into the cell cytoplasm across the outer and inner membranes by iron-repressible outer-membrane protein (IROMP) receptors and ATP-binding cassette

Abbreviations: DAP, 1,3-Diaminopropane; DPD, 2,2'-dipyridyl; Fur, ferric uptake regulator; FURTA, Fur titration assay; IROMP, iron-repressible outer-membrane protein; MFS, major facilitator superfamily; OMP, outer-membrane protein.

The GenBank/EMBL/DDBJ accession number for the sequence reported in this paper is AB661448.

A supplementary figure and a supplementary table are available with the online version of this paper.
transport systems or major facilitator superfamily (MFS) transporters, respectively. (Braun et al., 1998; Andrews et al., 2003; Cuı́v et al., 2004). The receptors display relatively tight specificity for their cognate siderophores. Translocation of a ferric siderophore through the receptor into the periplasmic space is dependent on the energy-transducing TonB system, comprising two cytoplasmic membrane proteins, ExbB and ExbD, in addition to TonB, which traverses the periplasmic space (Letain & Postle, 1997). The receptors commonly possess a conserved sequence, the TonB box, which interacts with TonB to gain the energy necessary to transport the ferric siderophore into the periplasm. Most siderophores can be classified into three major groups, i.e. catecholate, hydroxamate and α-hydroxycarboxylate (Hider & Kong, 2010). The ability to synthesize and utilize siderophores has been shown to be one of the virulence determinants in numerous bacterial pathogens, since the siderophores can facilitate the acquisition of iron from iron-withholding proteins, such as transferrin and lactoferrin in animal hosts (Schaible & Kaufmann, 2004; Bullen et al., 2005; Weinberg, 2009). On the other hand, high intracellular concentrations of iron may damage bacteria through the formation of undesired reactive oxygen species. In Gram-negative bacteria, maintenance of iron homeostasis is mainly regulated by the ferric uptake regulator (Fur), which functions as a transcriptional regulator (Bagg & Neilands, 1987; Escolar et al., 1999). When the intracellular iron concentration is increased, Fur dimerizes with Fe²⁺ as a cofactor and binds to a 19 bp consensus sequence, termed the Fur box (de Lorenzo et al., 1987; Calderwood & Mekalanos, 1988), present in the promoters of genes generally involved in iron acquisition.

*Acinetobacter haemolyticus* has emerged as an environmental bacterium and an opportunistic, multidrug-resistant, intrahospital human pathogen, which causes septicemia, pneumonia, meningitis, skin and wound infections, and urinary tract infection (Bergogne-Bérézin & Towner, 1996). Recently, a comparative study on the virulence potential of *Acinetobacter* species has suggested that *A. haemolyticus* and *Acinetobacter baumannii* are likely to be the most hazardous species with regard to growth ability in the mammalian environment, toxicity and intracellular infectivity (Tayabali et al., 2012).

We have previously reported that, when grown under conditions of iron depletion, *A. haemolyticus* ATCC 17906<sup>T</sup> produces a citrate-based dihydroxamate siderophore, acinetoferrin (Fig. 1), which is characterized by 1,3-diaminopropane (DAP) moieties and monounsaturated acyl appendages (Okojo et al., 1994). In this regard, acinetoferrin is very similar in chemical structure to rhizobactin 1021 (Fig. 1) produced by *Sinorhizobium meliloti* (Persmark et al., 1993), in which the relevant biosynthesis gene cluster has been identified and characterized (Lynch et al., 2001). This study was undertaken to obtain insight into the genetic basis of the acinetoferrin-mediated iron uptake system of *A. haemolyticus* ATCC 17906<sup>T</sup>. The Fur titration assay (FURTA) (Stojilkovic et al., 1994) was used to identify a cluster of Fur-regulated genes involved in acinetoferrin biosynthesis and transport. We describe the identification and characterization of an operon consisting of eight ORFs, whose functions were elucidated by homology search and mutation-complementation analysis.

**METHODS**

**Bacterial strains, plasmids, oligonucleotides and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Sequences of all oligonucleotides used are presented in Table S1. *A. haemolyticus* ATCC 17906<sup>T</sup> and its mutants and *Escherichia coli* were routinely grown with shaking at 30 and 37 °C, respectively, in Luria–Bertani (LB) medium or on LB agar plates (1.5 % agar) containing 0.5 % NaCl. To impose iron limitation, the LB medium was supplemented with the iron chelator 2,2'-dipyridyl (DPA) at 150 μM. Media with and without DPA were named −Fe medium and +Fe medium, respectively. As necessary, appropriate antibiotics were added to the growth media at the following concentrations: ampicillin (50 μg ml⁻¹), apramycin (50 μg ml⁻¹) and tetracycline (10 μg ml⁻¹).

**FURTA.** The FURTA was performed as described elsewhere (Stojilkovic et al., 1994). *A. haemolyticus* ATCC 17906<sup>T</sup> chromosomal DNA fragments (0.5–1 kb) partially digested with Sau3AI were ligated into the BamHI site of pBluescript II KS(−), and the resulting plasmids were transformed into *E. coli* H1717. Transformants were incubated at 37 °C for 15–24 h on MacConkey lactose agar plates (Difco) containing 0.1 mM FeCl₃ and ampicillin (50 μg ml⁻¹). Red colonies (lac⁺) denote a FURTA-positive phenotype and indicate binding of the Fur–Fe<sup>3+</sup> complex to the promoter region transformed in the indicator strain. Plasmid DNA was isolated, and nucleotide sequences of the inserts were determined.

**Growth assays.** *A. haemolyticus* ATCC 17906<sup>T</sup> and its mutant strains were grown overnight in LB medium, and aliquots of the preculture were inoculated into 5 ml fresh LB medium at OD₆₀₀ 0.005 and

![Fig. 1. Chemical structures of acinetoferrin (a) and rhizobactin 1021 (b).](http://mic.sgmjournals.org)
shaken at 70 r.p.m. The OD600 was measured with an Advantec TVS062CA biophotorecorder (Advantec Toyo).

DNA manipulation, nucleotide sequencing, and protein sequence analysis. Standard DNA manipulations were carried out as described by Sambrook et al. (1989). Chromosomal DNA and plasmid DNA were extracted using a Wizard Genomic DNA Purification kit (Promega) and a High Pure Plasmid Isolation kit (Roche Diagnostics), respectively. Restriction enzymes were purchased from Roche Diagnostics. A Ligation-Convenience kit (Wako Pure Chemical Industries) was used for the DNA ligation reaction. DNA fragments were purified from agarose gels using a Pure Chemical Industries) was used for the DNA ligation reaction. DNA fragments were purified from agarose gels using a Plasmid Purification kit (Promega) and a High Pure Plasmid Isolation kit (Roche Diagnostics). Transmembrane helices in ActC and ActD were predicted using the HMMTOP 2.0 server (http://www.enzim.hu/hmmtop/html/adv_submit.html) (Tusnady & Simon, 2001).

RNA isolation and RT-PCR. A. haemolyticus ATCC 17906T was grown in LB broth to an OD600 of 0.3. The culture was split into two aliquots; one was left untreated to prepare + Fe cells, and the other was supplemented with DPD at 150 μM to prepare −Fe cells. Both aliquots were further incubated until an OD600 of 0.5 was reached. Total RNA was extracted from each cell pellet using an RNase-free DNase I (Ambion) to exclude possible contamination with traces of chromosomal DNA. RT-PCR was carried out with a ReverTra Dash RNA PCR kit (Toyobo), according to the manufacturer’s instructions. For first-strand cDNA synthesis, 1 μg pretreated total RNA was incubated in a total volume of 20 μl at 42°C for 1 h with the primer AfRT-R (Tayebi et al., 1998) to the internal sequence of actD. Subsequent PCR was performed with 1 μl of reverse transcriptase reaction mixture using the primer pairs designed for each gene to be tested. PCR conditions were as follows: after an initial denaturation of 2 min at 94°C, DNA was amplified for 30 cycles, with each cycle consisting of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min. Total RNA without treatment with the M-MLV reverse transcriptase was used as a negative control reaction for PCR to confirm the lack of genomic DNA contamination. 16S rRNA was used as an established endogenous internal control. The

Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant features</th>
<th>Source or reference</th>
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<tbody>
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<td><strong>A. haemolyticus strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 17906T</td>
<td>Clinical isolate from human sputum, type strain</td>
<td>ATCC</td>
</tr>
<tr>
<td>TF-achA ATCC 17906T, achA::aacC4 derivative; Apra'</td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>TF-actA ATCC 17906T, actA::aacC4 derivative; Apra'</td>
<td>This study</td>
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<tr>
<td>TF-actB ATCC 17906T, actB::aacC4 derivative; Apra'</td>
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<tr>
<td>TF-actC ATCC 17906T, actC::aacC4 derivative; Apra'</td>
<td>This study</td>
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<td>TF-actD ATCC 17906T, actD::aacC4 derivative; Apra'</td>
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<td><strong>E. coli strains</strong></td>
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<td>DH5α</td>
<td>endA1 hsdR17 (F' mcrA mcrB mrr) supE44 thi-1 recA1 gyrA96 relA1 Δ(argF-lacZYA)U169 deoR ([&amp;804 lac õ(lacZ)M15])</td>
<td>Promega</td>
</tr>
<tr>
<td>H1717</td>
<td>araD139 rpsL150 Δ(argF-lac) relA1 U169 fbb5301 deoC1 ptsF25 rbsF25 rboB fbin::Ap lacMu; host strain for FURTA</td>
<td>Stojiljkovic et al. (1994)</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td></td>
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<tr>
<td>pBluescript II KS(+)</td>
<td>High-copy-number cloning vector; Ap'</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pRK415</td>
<td>Broad-host-range plasmid; Tc'</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pFURTA-Af</td>
<td>Initially isolated FURTA-positive clone; pBluescript II KS(+) containing a 915 bp SalAI-SalAI fragment from ATCC 17906T; Ap'</td>
<td>This study</td>
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<td>pBC-actf</td>
<td>pBluescript II KS(+) containing a 2935 bp PstI–PstI fragment from ATCC 17906T; Ap'</td>
<td>This study</td>
</tr>
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<td>pBC-act2</td>
<td>pBluescript II KS(+) containing a 3609 bp XbaI–XbaI fragment from ATCC 17906T; Ap'</td>
<td>This study</td>
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<td>pRK415-act3</td>
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<td>pRK415-actB</td>
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<td>pRK415 containing the actC gene from ATCC 17906T; Tc'</td>
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<td>pRK415-actD</td>
<td>pRK415 containing the actD gene from ATCC 17906T; Tc'</td>
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proteins 16s-F and 16s-R were designed according to the nucleotide sequence of *A. haemolyticus* ATCC 17906T 16s rRNA (accession no. Z93437). RT-PCR products were detected in a 1.5% agarose gel stained with ethidium bromide and visualized in a Gel Doc XR (Bio-Rad).

**Outer-membrane protein (OMP) analysis.** Cells of ATCC 17906T and mutant strains grown for 12 h in +Fe and −Fe media were harvested by centrifugation. The OMP-rich fractions were prepared and analysed by SDS-PAGE as previously described (Yamamoto et al., 1995a). Protein concentrations were determined by the method of Markwell et al. (1978). The developed gel was stained with Coomassie brilliant blue R-250 followed by visualizing with a Gel Doc XR system (Bio-Rad). The IROMPs were electroblotted onto a pre-wetted PVDF membrane (ProBlott; Applied Biosystems) with a Trans-Blot semi-dry electrophoretic transfer cell (Bio-Rad) for determination of their N-terminal amino acid sequences by automated Edman degradation sequence of *A. haemolyticus* ATCC 17906T strain using primer pairs (AF and AR, and BF and BR; Table S1) for construction of amplicons A and B, respectively, of each target gene. On the other hand, the apramycin cassette was amplified using primer pairs appropriately designed to link amplicons A and B to the 5′ and 3′ sites of the *aacC4* cassette derived from vector pJG1011 (Gomez & Bishai, 2000) as a template. The AR and BF primers contained an extension of about 20 nt complementary to the *acc-F* and *acc-R* primers, respectively. The three PCR products purified were mixed and subjected to PCR-driven overlap extension (Heckman & Pease, 2007) with the AF and BR primers and a KOD-Plus-DNA polymerase (Toyobo). The purified PCR product containing the target gene inactivated by the *aacC4* cassette was introduced into the ATCC 17906T strain by electroporation according to the procedure of Leahy et al. (1994). Recombinant mutants, whose wild-type genes were replaced by allelic exchange via double-crossover recombination, were selected on LB agar plates containing apramycin. Candidate clones were examined by PCR with the AF and BR primers to verify gene replacement (data not shown). The resulting mutants were named TF-acbA, -actA, -actB, -actC and -actD (Table 1), and grown in the presence of apramycin. To provide the five above-mentioned mutated genes with *in trans* complementation, the plasmids pRK415-acbA, pRK415-actA, pRK415-actB, pRK415-actC and pRK415-actD were constructed by PCR using *A. haemolyticus* ATCC 17906T genomic DNA with the comp-F and comp-R primer sets (Table S1), all of which contained the corresponding entire genes. These plasmids were introduced into the respective mutant strains by electroporation (Leahy et al., 1994). Empty strains containing only pRK415 were also constructed. Mutants containing pRK415 were grown in the presence

### Table 2. Proteins with homology to products of the eight genes in the acinetobactin biosynthesis and transport operon of *A. haemolyticus* ATCC 17906T

<table>
<thead>
<tr>
<th>ORF (aa)</th>
<th>Homologous protein (aa)</th>
<th>Micro-organism</th>
<th>Identity/similarity (%) (aa overlap)</th>
<th>Accession no.</th>
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<td>acbA (594)</td>
<td>Putative siderophore biosynthesis protein (517)</td>
<td><em>A. baumannii</em> ATCC 17978</td>
<td>78/88 (517)</td>
<td>NC009085</td>
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<td>Rhizobactin biosynthesis protein, RhbC (585)</td>
<td><em>S. mellitii</em> 1021</td>
<td>34/53 (546)</td>
<td>AE006469</td>
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<td>acbB (493)</td>
<td>Putative Lys/Orn N^6^-monoxygenase (435)</td>
<td><em>A. baumannii</em> ATCC 17978</td>
<td>49/63 (443)</td>
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<td>Rhizobactin biosynthesis protein, RhbE (456)</td>
<td><em>S. mellitii</em> 1021</td>
<td>41/57 (451)</td>
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<td>acbC (605)</td>
<td>Putative IucA/IucC (813)</td>
<td><em>Anaobana variabilis</em></td>
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<td>Rhizobactin biosynthesis protein, RhbF (601)</td>
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<td>acbD (212)</td>
<td>Putative acetyltransferase (168)</td>
<td><em>A. baumannii</em> ATCC 17978</td>
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<td>37/54 (170)</td>
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<td>actC (411)</td>
<td>MFS protein (408)</td>
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<td>MFS protein (406)</td>
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<td>actA (752)</td>
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<td>Putative arabinose efflux permease (381)</td>
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<td>Permease of MFS (395)</td>
<td><em>Paenibacillus polymyxa</em> E681</td>
<td>30/54 (386)</td>
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of tetracycline and apramycin. All final constructs were sequenced to confirm that the sequences were correct.

**HPLC analysis of acinetoferrin.** Production of acinetoferrin was examined by HPLC with a Hitachi LaChrom Elite HPLC system equipped with an Inertsil C8-3 reversed-phase column (150 × 4.6 mm, 5 µm; GL Sciences). ATCC 17906<sup>T</sup> and its mutant strains were grown to OD<sub>600</sub> 0.4 in 50 ml of a chemically defined Tris-buffered succinate medium (pH 7.4) containing 0.1 mM FeCl<sub>3</sub> (Okujo et al., 1994), and culture supernatants filtered with 0.22 µm pore-size cellulose acetate filters were adjusted to pH 2.0 with solid citric acid. Acinetoferrin in the culture supernatants (50 ml) was adsorbed on an Amberlite XAD-7 column (120 × 10 mm ID, 20–60 mesh; Sigma-Aldrich), washed with 50 ml distilled water, and desorbed with 50 ml methanol. The methanol eluate was evaporated to dryness. The residue was resuspended in 1 ml methanol and a 10 µl aliquot was injected into the HPLC system for analysis. Forty-five per cent acetonitrile in 0.1 % trifluoroacetic acid and 54 % acetonitrile in 0.1 % trifluoroacetic acid were used as mobile phases. The gradient was as follows: 45 % acetonitrile for 5 min and then from 45 % to 54 % acetonitrile within 30 min, and held at 54 % acetonitrile for 10 min. Detection was at 220 nm with a flow rate of 0.5 ml min<sup>−1</sup>.

**RESULTS**

**Isolation of the DNA region encoding acinetoferrin biosynthesis and transport genes**

The FURTA system (Stojiljkovic et al., 1994) was successfully used to isolate Fur box-containing gene fragments from the *A. haemolyticus* ATCC 17906<sup>T</sup> chromosome. Out of the positive clones, one clone named pFURTA-Af (Table 1, Fig. S1) was found to contain a fragment, part of which (915 bp) was predicted to encode a product that was 73 % identical to

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**Fig. 2.** Nucleotide sequences in the intergenic regions of the acinetoferrin biosynthesis and transport genes in *A. haemolyticus* ATCC 17906<sup>T</sup> and the partial deduced amino acid sequence of ActA. Acinetoferrin cluster genes were also identified as belonging to an iron-regulated operon by RT-PCR. (a) The putative –10 and –35 promoter elements, start codons (in bold type) and stop codons are presented. The putative Fur box sequence is underlined with nucleotide matches with the 19 bp *E. coli* consensus Fur box sequence. The terminator signal is indicated by opposing arrows, and the amino acid sequence deduced from actA, which is compatible with the N-terminal sequence determined for the ActA protein, is indicated by double underlines. Numbers correspond to sequence positions in GenBank accession number AB661448. (b) Lanes contain RT-PCR amplicons amplified from total RNA isolated from –Fe and +Fe cells of *A. haemolyticus* ATCC 17906<sup>T</sup>. The sizes of the amplicons derived from the representative genes are as follows: acbA, 334 bp; actA, 368 bp; and actD, 300 bp. As an internal control, a 225 bp fragment of the 16S rRNA gene was included. Lanes +RT and –RT represent RT-PCR products with and without reverse transcriptase, respectively; lane M, molecular size standard.
A1S_1647, one of the biosynthetic enzymes for a predicted hydroxamate siderophore produced by *A. baumannii* strains (Antunes et al., 2011; Eijkelkamp et al., 2011), and was 27% identical to RhbC, one of the biosynthetic enzymes for rhizobactin 1021 produced by *S. meliloti* (Lynch et al., 2001). As expected, the potential Fur box, which matched 15 of 19 nt of the consensus Fur box (de Lorenzo et al., 1987; Calderwood & Mekalanos, 1988), was detected in the promoter region. Then, based on these findings, the DNA sequence of the 13.1 kb region was determined, identifying eight ORFs (Fig. S1a). Cloning and sequencing of this region were performed using four plasmids (pBC-acf1, pBC-acf2, pRK-acf3 and pRK-acf4) with overlapping DNA fragments (Fig. S1b) which had been isolated by colony hybridization with DIG-labelled probes (Fig. S1c).

**Predicted protein sequences**

The deduced amino acid sequences of eight ORFs shared significant homology to known or predicted siderophore biosynthetic enzymes and transporters in other bacteria (Table 2). The presence of ORFs that encode proteins homologous to putative ω-amino acid monoxygenases, acetyltransferases and TonB-dependent siderophore receptors implied a cluster associated with biosynthesis and transport of the hydroxamate siderophore, acinetoferrin. Protein products of two genes, *orf9* and *orf10*, not linked to the preceding gene, were homologous to a putative IS4 family transposase ORF1 and hypothetical protein, respectively, in *A. baumannii* strains, suggesting that they were not involved in acinetoferrin-mediated iron acquisition. On the basis of potential functions inferred from homology, the biosynthetic and transport genes were named *acbABCD* (*acb* stands for acinetoferrin biosynthesis) and *actBCAD* (*act* stands for acinetoferrin transport; Fig. 2a). The predicted amino acid sequences of AcbABCD shared 34–41% identity with those of the *S. meliloti* rhizobactin 1021 RhbCDEF (Lynch et al., 2001), in keeping with the structural similarities between the siderophores produced by these species (Fig. 1). However, the

![Fig. 3. HPLC profiles of acinetoferrin secreted by the wild-type *A. haemolyticus* ATCC 17906T strain and deletion mutants into culture supernatants. The growth and HPLC conditions are described in Methods. A 10 μl aliquot of the acinetoferrin extract (1 ml) was injected into the HPLC instrument. The peak with a retention time of 19.5 min was collected and identified as acinetoferrin by MS analysis.](http://mic.sgmjournals.org)
gene order in *A. haemolyticus* was not the same as that in *S. meliloti*. Moreover, unlike in *S. meliloti*, the *A. haemolyticus* acinetoferrin cluster did not contain genes involved in DAP biosynthesis. Interestingly, *A. haemolyticus* AcbABD proteins (excluding AcbC) were highly similar (49–77% identity, 63–87% similarity) to the corresponding enzyme proteins in eight fully sequenced *A. baumannii* genomes, e.g. A1S_1647, _1648, and _1657 in *A. baumannii* ATCC 17978 (Eijkelkamp et al., 2011), whereas the arrangement of these genes in *A. baumannii* strains was quite different from that in the ATCC 17906‡ strain. An AcbC homologue was not found in any of the whole-genome-sequenced *A. baumannii* strains.

The product of *actA* showed a significant similarity with various TonB-dependent outer-membrane receptors for ferric siderophores, including RhtA for ferric rhizobactin 1021 in *S. meliloti* (Lynch et al., 2001). The amino acid sequence in the N-terminal region deduced from *actA* was identical to the first 10 N-terminal amino acids determined for the 79.3 kDa IROMP (see below). The product of *actB* showed homology to hypothetical proteins whose functions have not been ascertained for any species. The product of *actC* showed 61% identity to bacterial MFS proteins (Table 2). It was noteworthy that ActC also showed significant homology to the *S. meliloti* RhtX, an inner-membrane importer for ferric rhizobactin 1021 subsequent to outer-membrane translocation (Cuı´ v et al., 2004). HMMTOP analysis (Tusnády & Simon, 2001) placed ActC within the group of inner-membrane proteins with 12 transmembrane domains belonging to the MFS. ActD, the last gene product, showed homology to MFS efflux permeases and was divided into an inner-membrane protein with 12 transmembrane domains by HMMTOP analysis. Its subcellular location and similarity to MFS

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**Fig. 4.** Growth profiles of *A. haemolyticus* ATCC 17906‡, TF-actA, TF-actA/pRK415 (empty) and TF-actA/pRK415-actA strains. The ATCC 17906‡ (○), TF-actA (●), TF-actA/pRK415 (▼) and TF-actA/pRK415-actA (▲) strains were grown in –Fe medium. The OD₆₀₀ was measured every 2 h for 24 h. A representative of three independent experiments is shown.

**Fig. 5.** OMP profiles of *A. haemolyticus* ATCC 17906‡, TF-actA, TF-actA/pRK415-actA and TF-actA/pRK415 strains. All strains were grown in +Fe and –Fe media for 12 h. SDS-PAGE was carried out using a 7.5% polyacrylamide running gel (130 mm) and the gel was stained with Coomassie brilliant blue R-250. The amount of protein loaded in each lane was 20 μg. Arrowheads in lanes 3 and 5 indicate the 79.3 kDa IROMP. Lanes 1 and 3, *A. haemolyticus* ATCC 17906‡; lanes 2 and 4, TF-actA; lane 5, TF-actA/pRK415-actA; lane 6, TF-actA/pRK415; lane M, molecular mass marker proteins.

**Fig. 6.** Complementation of the *act* mutants with their corresponding intact genes. TF-actA, TF-actB, TF-actC and TF-actD strains and these mutants complemented with the plasmids pRK415-actA, pRK415-actB, pRK415-actC and pRK415-actD, respectively, were grown in –Fe medium, and their OD₆₀₀ values were compared after a 24 h incubation. As a control, mutants containing the empty plasmid pRK415 were grown under the same conditions. Data are the means from three independent experiments; error bars, sd. P values were estimated by Student’s *t* test (*, *P* < 0.05).
proteins suggested that ActD could be involved in acinetoferrin secretion. Finally, although there were no proven functions for actB homologues in the three bacterial species cited, we note that all of them were located upstream of putative MFS importer genes followed by putative ferric siderophore outer-membrane receptor genes in the same order as actB in the ATCC 17906T strain.

The acbABCD and actBCAD genes form an iron-regulated operon

The eight genes were located consecutively and oriented in the same direction with a single set of putative promoter elements in front of acbA and with a single rho-independent transcription terminator sequence just downstream of the stop codon of actD, suggesting that the eight genes are transcriptionally linked (Fig. 2a). This transcriptional organization was also suggested by the overlaps that occurred at the gene junctions as well as the presence of relatively short intervening sequences without any promoter elements. Then, in order to confirm that this gene cluster was organized as an iron-regulated operon, RT-PCR analysis was performed for acbA, actA and actD with three different primer combinations (Table S1) that amplified internal fragments of the corresponding genes. The primer (AFRT-R) complementary to the internal sequence of the last gene, actD, was used for the first-strand cDNA synthesis. Each of the reactions yielded an amplicon of the expected size without interference from DNA contamination, only when total RNA from cells grown under iron-limiting conditions was used (Fig. 2b). In addition, the level of 16S rRNA expression as a control was not affected by the iron conditions in the medium. In particular, the results of PCR for acbA and actD, the first and last genes within the eight-gene cluster, demonstrated that these genes were co-transcribed from the promoter upstream of acbA as a single polycistronic message under iron-limiting conditions. The presence of a potential Fur box in the promoter region of acbA implied that Fur was responsible for this iron-regulated transcription.

Phenotypic analysis of the acbA mutant

In order to characterize the acinetoferrin biosynthesis cluster, the first gene, acbA, was mutated using gene replacement with insertion (Aranda et al., 2010). No discernible growth defect was observed for the acbA mutant strain when grown in +Fe medium (data not shown). As expected, A. haemolyticus ATCC 17906T produced acinetoferrin when grown in the –Fe medium alone, but the TF-acbA mutant defective in acbA did not produce acinetoferrin under the same conditions (Fig. 3). In contrast, the complementing strain, TF-acbA/pRK415-acbA, produced acinetoferrin at a level similar to that of the wild-type parental strain, indicating that the acbA gene was one of the determinants essential for acinetoferrin biosynthesis. Nevertheless, the acbA mutant could grow to a modest extent with a reduced growth rate in the –Fe medium but could not reach the same final OD600 as the wild-type strain (Fig. 4a). The culture supernatant of the acbA mutant exhibited a catecholate siderophore titre similar to that of the parental strain, as determined by the Arnow colorimetric assay (Payne, 1994). It was also positive in the Chrome azurol S liquid assay, which can monitor siderophore production (Schwyn & Neilands, 1987). These results were consistent with the report that the ATCC 17906T strain also produces a small amount of the catecholate siderophore acinetobactin, which is also produced by A. baumannii (Yamamoto et al., 1994; Wuest et al., 2009). Therefore, the modest growth observed for the acbA mutant may be attributable to acinetobactin. These results suggested that under iron-limiting conditions, acinetobactin could partially replace acinetoferrin in supplying iron to A. haemolyticus cells. However, the possibility was not ruled out that the ATCC 17906T strain can acquire iron, although to a lesser extent, via an uncharacterized siderophore, which is different from both acinetobactin and acinetoferrin but capable of promoting growth of this strain under iron-limiting conditions. On the other hand, the complementing strain, TF-acbA/ pRK415-acbA, had a reduced growth rate but eventually reached almost the same final OD600 as the parental strain.

Fig. 7. Proposed biosynthetic pathway for acinetoferrin.
(Fig. 4). The strain (TF-acbA/pRK415) containing an empty plasmid also showed a more reduced growth rate than the complementing strain. However, a highly reduced growth rate was observed for TF-acbA containing pRK415 or pRK415-acbA when it was grown in –Fe medium supplemented with both tetracycline and apramycin, although the reason for this is currently unknown. The growth assay at least indicated thatacbA participates in acinetoferrin production and that acinetoferrin was the preferred siderophore of this strain under the growth conditions used in this study. Taken together, these data indicate that A. haemolyticus ATCC 17906T produced a second siderophore, acinetobactin, but in an amount insufficient to grow at a level similar to the parental strain. Additionally, the acbA mutant showed normal expression of the ActA receptor protein, the seventh gene product (data not shown), confirming that the gene replacement mutation in acbA caused no polar effect on the downstream genes.

**Phenotypic analysis of the actA mutant**

A. haemolyticus ATCC 17906T was grown in +Fe and –Fe media, and the profiles of total outer-membrane proteins

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**Fig. 8.** Amino acid sequence alignment of A. haemolyticus ATCC 17906T ActC with the three proteins showing the highest homology and S. meliloti RhtX. The numbers refer to amino acid positions in the unprocessed protein. Amino acids identical in the five proteins are marked by asterisks, and conserved amino acids are denoted by colons. The accession numbers of the aligned proteins are as follows: Pectobacterium carotovorum PC1_3088 (NC012917), P. mendocina NK-01 MDS_4916 (CP002620), Stenotrophomonas maltophilia R551-3 Sma1_0074 (NC011071) and S. meliloti 1021 RhtX (AE006469). The four conserved amino acid residues that are predicted to be located in a cytoplasmic loop of a novel family of MFS permeases (Cuiv et al., 2004) are boxed.
(OMPs) were then compared by SDS-PAGE (Fig. 5). As a result, at least five IROMP bands were observed, only when the ATCC 17906<sup>T</sup> strain was grown in –Fe medium (Fig. 5, lanes 1 and 3). The first 10 N-terminal amino acid sequence of the 79.3 kDa IROMP (the predicted molecular mass of the mature ActA was 79 249 kDa) was identical to that deduced from actA (Fig. 2a). This also indicated that the 23 amino acid residues were removed from the ActA pre-protein as a signal peptide. The actA mutant TF-actA was constructed to determine loss of expression of the corresponding protein. As expected, the 79.3 kDa IROMP band disappeared in this mutant (Fig. 5, lanes 2 and 4), and the corresponding band was again detected in the complementing strain, TF-actA/pRK415-actA, although it was not detected in the mutant containing the empty plasmid (Fig. 5, lanes 5 and 6). Next, we determined the effect of defective actA on growth in the –Fe medium. TF-actA showed an approximate 50 % reduction in growth compared with the parental strain (Fig. 6), as observed for the acbA mutant (Fig. 4). In contrast, the actA mutant complemented with the actA gene outgrew the parental strain when incubated for 24 h under iron-limiting conditions (Fig. 6). Altogether, these data indicated that ActA could act as the outer-membrane receptor for ferric acinetoferrin.

**Phenotypic analysis of the actD mutant**

The inactivation of actD (TF-actD) resulted in an approximate 35% reduction in the ability to secrete acinetoferrin compared with the parental strain, whereas the introduction of pRK415-actD into the actD mutant (TF-actD/pRK415-actD) regained the strain’s original ability to secrete acinetoferrin (Fig. 3). In addition, TF-actD/pRK415-actD outgrew the actD mutant (Fig. 6). These results suggested that ActD may function as an inner-membrane exporter for acinetoferrin.

**Phenotypic analysis of the actB and actC mutants**

When TF-actB, TF-actC and their complementing strains (TF-actB/pK415-actB and TF-actC/pRK415-actC) were cultured in –Fe medium, no significant differences in the levels of growth after 24 h were observed (Fig. 6), and they were able to grow at levels similar to the parental strain (Fig. 4). Thus, mutational analysis failed to confirm the functions of actB and actC in ferric acinetoferrin transport. In particular, it was assumed that the additional production of acinetobactin could hamper the assessment of the function of ActC as an inner-membrane importer for ferric acinetoferrin.

**DISCUSSION**

The Fur box-containing DNA fragment isolated from A. haemolyticus ATCC 17906<sup>T</sup> by the FURTA method facilitated cloning and sequencing of neighbouring ORFs of related functions. Homology and mutational analyses revealed a cluster of eight ORFs encoding biosynthetic enzymes and transport components for acinetoferrin. We demonstrated that these eight ORFs were regulated by iron and were co-transcribed as a single polycistrionic message. The results reported here indicate that the biosynthesis of acinetoferrin in A. haemolyticus ATCC 17906<sup>T</sup> likely proceeds as shown in Fig. 7. This represents another example of the siderophore biosynthesis pathways independent of nonribosomal peptide synthetases (Oves-Costales et al., 2009).

It has been reported that neighbouring genes encoding RhbA (1-2,4-diaminobutyrate : 2-ketoglutarate 4-amino-transferase) and RhbB (1-2,4-diaminobutyrate decarboxylase), which are required for DAP production, are present as the first and second ORFs in the iron-regulated rhizobactin 1021 biosynthesis operon of S. meliloti (Lynch et al., 2001). Although such genes were not present in the acinetoferrin biosynthesis operon, significant activities of these two enzymes were found in the ATCC 17906<sup>T</sup> strain even when grown in +Fe medium (Yamamoto et al., 1995b; Ikai & Yamamoto, 1997). Moreover, there was a gene located at the same direction as acbA immediately upstream of acbA, whose protein product was homologous to hypothetical proteins of A. baumannii strains not related to DAP production. These data support the possibility that genes involved in DAP production may be located in another chromosomal region of the strain and that the transcription of these genes may not be iron-regulated. Consistent with this, it has recently been reported that A. baumannii, belonging to the same genus as A. haemolyticus, produces DAP, which is responsible for its surface-associated motility and virulence, even under iron-replete conditions (Skielbe et al., 2012). Besides rhizobactin 1021 (Persmark et al., 1993) and acinetoferrin, schizokinin (Mullis et al., 1971) and synechobactin (Ito & Butler, 2005) are known as hydroxamate siderophores characteristically containing DAP moieties for structural assembly. The results in this study would be useful to elucidate the genetic determinants involved in biosynthesis and transport of these siderophores.

To date, three different siderophore biosynthesis gene clusters have been described in A. baumannii (Antunes et al., 2011; Eijkelkamp et al., 2011). One gene cluster that is common to all the whole-genome-sequenced A. baumannii strains is proposed to function in biosynthesis of a hydroxamate siderophore of unknown structure. The acbA, acbB and acbD orthologues in the acinetoferrin biosynthesis cluster were found to be present in the hydroxamate siderophore cluster of A. baumannii. However, the genetic arrangement differs considerably between the two species, and A. baumannii has no acbC orthologue. These findings suggested that the predicted hydroxamate siderophore in A. baumannii may be structurally related to acinetoferrin.
The proteins *Pseudomonas aeruginosa* Ptx (Cuıv et al., 2004) and *Legionella pneumophila* LbtC (Chatfield et al., 2012), which show homology to *S. meliloti* RhtX, a member of a novel family of permeases (Cuıv et al., 2004), have also been reported to be encoded in the gene clusters that are involved in siderophore-mediated iron acquisition systems. The actC gene encoding the protein homologous to RhtX is located between actB and actA in the acinetoferrin cluster (Fig. 2a), and amino acid sequence alignment of these ActC homologues with RhtX revealed that a motif of four amino acids, QD(V/I)A, was conserved (Fig. 8), which is predicted to be located in a cytoplasmic loop in RhtX (Cuıv et al., 2004). These observations suggested that ActC may also be a member of a novel family of permeases and function in part as an importer of ferric acinetoferrin. Moreover, it is of interest that all of the genes encoding proteins with the highest similarity to ActC that are cited in Table 2 are preceded by genes encoding ActB homologues of unknown function. *Pseudomonas mendocina* has also been reported to produce a hydroxamate siderophore of unknown structure (Awaya & Dubois, 2008). The function of ActB in *A. haemolyticus* ATCC 17906T, however, remains obscure.

It is of great interest to elucidate how siderophores newly synthesized under iron-limiting conditions are secreted into the extracellular milieu, since intracellular accumulation of iron-free siderophores may be toxic due to deprivation of the cellular components of essential iron cofactors. MFS efflux pumps with 12 transmembrane segments in several bacteria, e.g. in *E. coli* (EntS) (Furrer et al., 2002), *Bordetella* species (AlcS) (Brickman & Armstrong, 2005), *Legionella pneumophila* (IbtB) (Allard et al., 2006) and *Vibrio parahaemolyticus* (PvsC) (Tanabe et al., 2006), have been identified as siderophore-specific exporters. This work indicated that *A. haemolyticus* ATCC 17906T also secretes acinetoferrin with the help of ActD. However, it should be considered that the actD mutant was still able to secrete acinetoferrin in response to iron limitation, although to a lesser extent than the wild-type parental strain (Fig. 3). This may be explained by compensation by another efflux pump in this species to avoid accumulation of acinetoferrin at burdensome levels, similar to *P. aeruginosa*, in which the siderophore pyoverdine is also exported by another known multidrug efflux pump (Poole et al., 1993).

Recently, we identified the gene cluster involved in biosynthesis and transport of acinetobactin in *A. haemolyticus* ATCC 17906T (GenBank accession no. AB621369), whose gene order is very similar to that in the characterized acinetobactin gene cluster of *A. baumannii* ATCC 19606T (Mihara et al., 2004). However, genes encoding an integrase catalytic subunit and a transposase IS3/IS911 family protein intervene in the 3′-terminal region of a thioesterase gene, thereby resulting in the absence of the C-terminal 24 amino acid residues. Deletion of angT, encoding a thioesterase which is involved in anguibactin biosynthesis in *Vibrio anguillarum*, has been reported to lead to a 17-fold decrease in its production (Wertheimer et al., 1999). Since anguibactin is structurally similar to acinetobactin, it was assumed that *A. haemolyticus* ATCC 17906T may also produce acinetobactin even in small amounts. For this reason, we attempted to generate a double mutant both in *acbA* and in the gene involved in acinetobactin biosynthesis. However, in spite of extensive efforts, such a mutant could not be obtained. Construction of a double mutant with regard to two different siderophore biosynthesis clusters may be necessary to clarify the advantages of possession of independent siderophore biosynthesis pathways in *A. haemolyticus*.

In conclusion, we have identified the *A. haemolyticus* ATCC 17906T gene cluster spanning ~13 kb which participates in acinetoferrin biosynthesis and transport. This cluster includes eight ORFs which are all co-transcribed from the promoter located in front of *acbA*. The existence of the iron acquisition system mediated by acinetoferrin would give *A. haemolyticus* the opportunity to utilize different iron sources, ultimately enhancing its ability to colonize and prosper in its natural habitat and human host. Further studies will be focused on identification and characterization of genes encoding the inner-membrane active transport system for ferric acinetoferrin in connection with the function of ActC.

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